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Cold Shock and Cold Acclimation Proteins in the Psychrotrophic Bacterium *Arthrobacter globiformis* SI55

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The psychrotrophic bacterium *Arthrobacter globiformis* SI55 was grown at 4 and 25°C, and the cell protein contents were analyzed by two-dimensional electrophoresis. Cells subjected to cold shocks of increasing magnitude were also analyzed. Correspondence analysis of protein appearance distinguished four groups of physiological significance. Group I contained cold shock proteins (Csps) overexpressed only after a large temperature downshift. Group II contained Csps with optimal expression after mild shocks. Group III contained proteins overexpressed after all cold shocks. These last proteins were also overexpressed in cells growing at 4°C and were considered to be early cold acclimation proteins (Caps). Group IV contained proteins which were present at high concentrations only in 4°C steady-state cells and appeared to be late Caps. A portion of a gene very similar to the *Escherichia coli cspA* gene (encoding protein CS7.4) was identified. A synthetic peptide was used to produce an antibody which detected a CS7.4-like protein (A9) by immunoblotting two-dimensional electrophoresis gels of *A. globiformis* SI55 total proteins. Unlike mesophilic microorganisms, this CS7.4-like protein was still produced during prolonged growth at low temperature, and it might have a particular adaptive function needed for balanced growth under harsh conditions. However, A9 was induced at high temperature by chloramphenicol, suggesting that CS7.4-like proteins have a more general role than their sole implication in cold acclimation processes.

Microbial growth involves a complex series of integrated chemical reactions which are directly affected by external temperature. The most susceptible cell components are proteins, since subtle changes in their configuration may result in their inactivation. Cellular proteins, particularly regulatory proteins and key metabolic enzymes, require adjustments to cope with the temperature shifts that occur in natural or artificial environments and to allow balanced growth at the new temperature. The effects of temperature on microbial growth can be expressed by the Arrhenius equation (12, 20). For most microorganisms, the slope of Arrhenius plots is linear over a wide temperature range (about 30°C). When temperature shifts are performed within this range, the growth rate usually changes rapidly, and the protein compositions of cells remain fairly constant. At temperatures above or below this range, the response is no longer linear and growth finally ceases. Rapid temperature shifts to the upper or lower limits have a pronounced effect on the physiology of bacterial cells, and the synthesis of specific sets of stress proteins is induced or enhanced (15).

The heat shock response is universal and has been thoroughly studied. The major heat shock proteins are highly conserved. They are involved in the homeostatic adaptation of cells to harsh environmental conditions. Some act as molecular chaperones for protein folding, while others are involved in the processing of denaturated peptides whose accumulation would be deleterious (14, 35, 46). A cold shock response that is common to both eukaryotic and prokaryotic microorganisms has recently been described (22). It involves the transient induction of a subset of proteins, the cold shock proteins (Csps). This response to cold has been extensively studied in the mesophilic bacterium *Escherichia coli* (8, 24). A temperature shift from 37 to 10°C results in a 4- to 5-h-long lag before growth resumes at a much reduced rate. During the lag period, the synthesis of most cellular proteins is repressed, and only 30 to 40 proteins can be detected by two-dimensional gel electrophoresis 2 h after cold shock treatment. Fourteen of these proteins are Csps, 10 of which have been identified: NusA (involved in termination and antitermination), RecA (DNA recombination and SOS response), H-NS (a nucleoid-associated DNA-binding protein), GyrA (the α subunit of topoisomerase DNA gyrase), initiation factor 2 of translation (subunits a and b), polynucleotide phosphorylase (degradation of mRNA), pyruvate dehydrogenase, dihydrolipoamide acetyltransferase (a subunit of pyruvate dehydrogenase), and CS7.4.

Only one of these proteins, CS7.4, the product of the *cspA* gene, is specifically produced after cold shock (9), and it accounts for 13% of the total cell protein. CS7.4 is a 70-residue peptide having five antiparallel sheets that form a β-barrel with no helical structure (31). CS7.4 harbors two RNA binding sequences, motifs RNP1 and RNP2, suggesting that CS7.4 acts as an RNA chaperone-like protein by unfolding tightly compacted molecules inaccessible for translation or by preventing their translation while protecting them from degradation (22). CS7.4 also acts as a transcriptional activator for at least two genes, one encoding Csp H-NS (29) and the other encoding GyrA (23). Both proteins are involved in the control of DNA superhelicity (7, 17). CS7.4 is very similar to the DNA binding domain of the Y-box transcription factors, i.e., DNA-binding proteins which bind specifically to inverted CCAAT box sequence elements located in some eukaryotic gene enhancers and promoters (28, 49). Proteins with up to 60% identity with CS7.4 have been found in *Bacillus subtilis* (CspB) (48) and *Streptomyces clavuligerus* (SC7.0) (2). Although the *E. coli* Csps

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have been thoroughly studied, their direct implication in the cold survival of mesophilic bacteria has not been demonstrated. Very few studies of the cold shock response in cold-adapted microorganisms, i.e., psychrotrophs and psychrophiles, have been done. Csps have been found mainly in the psychrotrophic yeast *Trichosporon pullulans* (25) and in the psychrotrophic bacterium *Bacillus psychrophilus* (47). In addition to Csps, psychrotrophic bacteria can express a second class of proteins, cold acclimation proteins (Caps), during balanced growth at low temperatures (13, 38, 47). These proteins have never been described for mesophilic bacteria, and they might play an important role in the physiology of cells during cold adaptation. No information on their structure or their function is yet available.

The aim of this work was therefore to examine the cold shock response and subsequent cold acclimation of the psychrotrophic bacterium *Arthrobacter globiformis* SI55. A mathematical approach using correspondence analysis (CA) was used to classify cold shock-induced proteins (Csps) into three groups of physiological significance. A fourth group contained proteins that were characteristic of life at low temperatures, i.e., late Caps. PCR was used to identify a portion of a gene with a high degree of similarity with the *cspA* gene of *E. coli*. The partial sequence of this gene was used to construct a synthetic peptide which was used to produce an antibody. A CS7.4-like protein was detected by immunoblotting of two-dimensional electrophoresis gels of *A. globiformis* SI55 total proteins. Unlike mesophilic microorganisms, the corresponding protein (A9) was still produced during prolonged growth at the low temperature, and it might serve a particular function necessary for cold acclimation.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *A. globiformis* SI55 was grown aerobically in a liquid synthetic medium to mid-exponential phase (optical density at 570 nm, 0.5) as previously described (39). An Arrhenius plot (see Fig. 1) was established by monitoring growth temperatures from 4 to 32°C and determining specific growth rates. Experiments with chloramphenicol were conducted at 25°C in complete medium (pH 7.0) containing (per liter) 5.0 g of biotrypticase, 2.5 g of yeast extract, 5 g of glucose, 1 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$, and 0.1 ml of oligoelements. The oligoelement solution consisted of 1 g of $FeCl_3 \cdot 6H_2O$, 0.5 g of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g of $ZnSO_4 \cdot 7H_2O$, 0.3 g of $MnSO_4 \cdot H_2O$, 0.5 g of H_3BO_3, and 0.0002 g of $CoCl_2 \cdot 6H_2O$ in 100 ml of distilled water.

Csp radiolabelling and sample preparation. Cold shock was accomplished by shifting 1 ml of cells growing exponentially at 25°C (optimal growth temperature) in precooled test tubes (13 by 100 mm) to the temperatures indicated below for 1 to 6 h. The set temperature was reached within 2 min after transfer. The growth of cold-shocked cells was monitored spectrophotometrically at 570 nm. Proteins were radiolabelled for two-dimensional electrophoresis by adding 10.25 μCi (3.79 MBq) of L-[^{35}S]methionine (specific activity, 42.2 TBq $mmol^{-1}$) to each tube for the last 30 min of incubation at the appropriate temperature. Cells were rapidly harvested by centrifugation into precooled Eppendorf tubes, and methionine incorporation was stopped by adding 1 ml of acetone to the cell pellet. The acetone was removed by centrifugation, and the pellet was air dried. The cells were resuspended in 200 μl of O'Farrell (33) lysis buffer, and cells walls were disrupted by sonication (Braun Labsonic; 50 W) for 8 min (30-s periods). Unbroken cells were removed by centrifugation at 12,000 × *g* for 5 min. Samples were stored at −80°C.

Acid-insoluble radioactivity was determined by spotting 2 μl of cell extracts onto glass fiber filters (Whatman GF/C), which were then rinsed with 5 ml of cold 25% (vol/vol) trichloracetic acid (TCA), 10 ml of cold 10% (vol/vol) TCA, and then 10 ml of 96% (vol/vol) ethanol. The filters were placed in 4 ml of Aqualyte scintillation cocktail (Baker Chemical), and the precipitated radioactivity was determined.

Radiolabelling of Caps. The electrophoretic patterns of cells growing exponentially at 25 and 4°C were compared to determine if *A. globiformis* SI55 overexpressed certain proteins during continuous growth at low temperature. Cells were grown at these two temperatures and radiolabelled during mid-logarithmic growth (optical density at 570 nm, 0.5), essentially as described for cold shock radiolabelling. Cells were incubated with L-[^{35}S]methionine for 30 min at 25°C and for 4 h at 4°C, periods which correspond to the same fraction of their generation times.

Two-dimensional electrophoresis. Isoelectric focusing was done in rod gels (12 by 1.5 mm) as described by O'Farrell (33). The gel mixture was 4% (wt/vol) acrylamide–9.2 M urea–2% Nonidet P-40–5% carrier ampholyte ampholines (LKB) (1 part pH 3.5 to 10, 2 parts pH 5 to 7). Samples (20 μl) containing 10^6 cpm were loaded on the basic side of the gels, and isoelectric focusing was performed at 10,000 V · h. The second dimension was standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system of Laemmli (27). Gels from the first dimension were incubated in denaturing buffer for 20 min and placed on top of a 1.5-mm-thick 12% polyacrylamide slab gel overlaid with a 4% polyacrylamide stacking gel. Electrophoretic separation was performed at 15°C at a constant 15-mA current per slab gel until the tracking dye entered the resolving gel, at which time the current was increased to 30 mA. The gels were stained with 1% (wt/vol) Coomassie blue R-250 in 50% (vol/vol) methanol–7.5% (vol/vol) acetic acid and destained with 30% (vol/vol) ethanol–10% (vol/vol) acetic acid. Autoradiograms were obtained by exposing dried gels to Kodak X-ray films.

An alpha-numeric code was assigned to the proteins whose expression increased after the temperature shift. A letter (A to G) was assigned to each peptide according to its isoelectric point (from the acid to the basic side of the gels), followed by a number corresponding to the estimated molecular weight (in thousands). The pH gradient was monitored by using two-dimensional electrophoresis standards (Bio-Rad) and by measuring the pH along the first-dimension gels. Molecular weights were estimated by the comigration of standard proteins in the second dimension. The molecular weight standards were rabbit muscle phosphorylase *b* (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and hen egg white lysozyme (14,400). In order to exclude from the analysis variations in the protein patterns due to artifacts related to the two-dimensional electrophoresis method, at least five gels from independent cultures and protein extracts at each temperature were analyzed. Only peptides that were reproducibly overexpressed were considered.

CA. CA (10) is commonly used to identify structures in a set of biological data (6). It is a multivariate method used to analyze contingency tables. The contingency table used in this paper was derived from Table 2 by summing the occurrences (i.e., the number of plus signs) of each protein (i.e., the rows) at each cold shock amplitude (i.e., columns) independently from their kinetics of appearance. CA was performed by using Analysis of Environmental Data (ADE) software 4.0 (44). In this study, the purposes of the CA were (i) to group proteins with similar distributions of occurrence in the different cold shocks (see factor map of rows, Fig. 4A) and (ii) to group the cold shocks responsible for the induction of the same proteins (see factor map of columns, Fig. 4B). Without using CA, such typologies are subjective and difficult to find, considering the dimensions of Table 2. The main advantage of CA is to group data patterns according to a statistical criterion based on the measure of the mathematical distance between points. The mathematical aspects of CA have been detailed elsewhere (see references 3, 18, 19, 26, and 32 for reviews).

Oligonucleotide primers. DNA amplification of the *A. globiformis* SI55 *cspA*-like region was done with two primers (A and B). They were designed to span two conserved regions of the *B. subtilis cspB* gene (48) (primer A, 5′-GTAAAG TAAAATGGTTCAAC-3′; and primer B, 5′-AGAAATGAACGAATACATC -3′). *A. globiformis* SI55 DNA was amplified with primers A and B, generating an 80-bp DNA fragment. A *Hin*dIII restriction site was generated in primer A, and an *Eco*RI restriction site was generated in primer B (A′, 5′-GTAAAGTA AA**AAGCTT**CAAC-3′; and B′, 5′-AGAAATGAAC**GAATTC**ATC-3′) in order to clone the PCR-amplified fragment.

DNA extraction and purification. Exponentially growing *A. globiformis* SI55 cells were harvested by centrifugation at 12,000 × *g* for 10 min. The pellet was washed twice with TKM buffer (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 50 mM KCl) and resuspended in this buffer (2 ml of TKM buffer per g [wet weight]). An equal volume of 0.10- to 0.11-mm-diameter glass beads was added, and the cells were disrupted in a Braun MKS grinder for 2 min at maximal speed. Overheating was prevented by cooling with liquid CO_2 for 10 s every 20 s. The glass beads were removed by centrifugation at 5,000 × *g* for 5 min. Unbroken cells and residual glass beads were then removed by centrifugation at 12,000 × *g* for 20 min. Total genomic DNA was purified by the procedure of Brenner et al. (4).

PCR amplification. PCR amplification was performed with a total volume of 50 μl in 0.5-ml Eppendorf tubes under a layer of paraffin oil in a DNA thermal cycler (Trio-thermoblock TB-1; Biometra, Göttingen, Germany) (30). The PCR was run for 35 cycles, during which DNA was denatured at 95°C for 1 min, primers were annealed at 50°C for 1 min, and DNA was extended at 72°C for 1 min. PCR-amplified DNAs were detected by horizontal 2% (wt/vol) agarose gel electrophoresis run in TBE buffer (0.089 M Tris borate, 0.0002 M EDTA; pH 8) at 10 V cm^{-1} for 90 min. The gels were stained in aqueous ethidium bromide (0.4 mg · $liter^{-1}$), rinsed with distilled water, and photographed with Ilford HP5 film and a 312-nm-UV source.

Cloning of the amplified DNAs. PCR products were cleaved with *Hin*dIII and *Eco*RI (Boehringer, Mannheim, Germany) and ligated into the corresponding restricted pBluescript II-SK$^-$ vector (Stratagene, La Jolla, Calif.), using 1 U of T4 DNA ligase (Boehringer). Plasmids were purified from the *E. coli* DH5α (Gibco BRL, Gaithersburg, Md.) host strain by alkaline lysis with a Midi-prep kit (Qiagen, Hilden, Germany). DNA was sequenced with a T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden) (42).

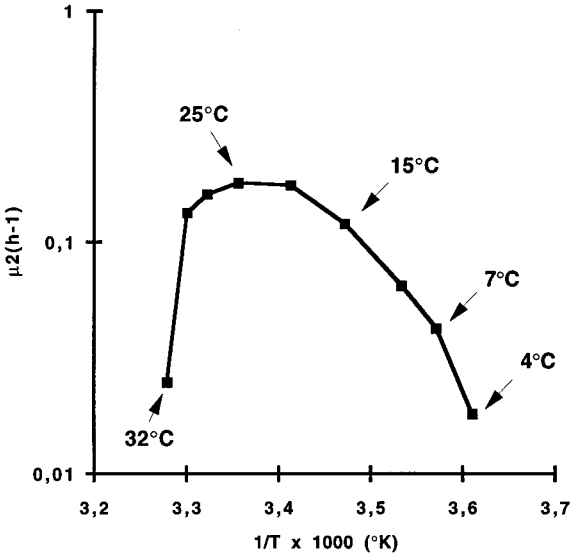

FIG. 1. Arrhenius plot of growth rates of *A. globiformis* SI55. Cells were grown at temperatures from 32 to 4°C in a liquid synthetic medium, and the specific growth rate was computed. The log of the specific growth rate constant μ (per hour) was plotted against the reciprocal of absolute temperature (K). Some datum points are marked with the corresponding temperature in degrees Celsius.

Antibody preparation and immunoblotting. A 14-residue synthetic peptide corresponding to the *A. globiformis* SI55 peptide sequence deduced from the *cspB*-CS7.4-like cloning region and conjugated to ovalbumin was purchased from Neosystem (Strasbourg, France). Antiserum against this synthetic peptide was obtained from Valbex (Villeurbanne, France). *A. globiformis* SI55 total proteins were separated by two-dimensional electrophoresis and transferred to Optitran BA-S85 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in a semi-dry electrophoretic transfer cell (Cera-labo, Aubervilliers, France). Transfer was done at 0.8 mA/cm^2 in 25 mM Tris-HCl (pH 8.3)–20% (vol/vol) methanol–0.15 M glycine. Membranes were blocked by being soaked in 5% (wt/vol) dried milk in Tris-buffered saline (TBS) (20 mM Tris [pH 7.5], 500 mM NaCl) for 2 h at 20°C and washed three times with 0.05% Tween 20 in TBS. Blots were incubated with the antiserum and then with goat anti-rabbit antibodies conjugated to alkaline phosphatase. The blots were stained with 5-bromo-4-chloro-3-indolylphosphate of *p*-toluidine and *p*-nitroblue tetrazolium chloride (Sigma Chemical, Saint Quentin Falavier, France) as described by the manufacturer.

Induction of a CS7.4-like protein with chloramphenicol. *A. globiformis* SI55 was grown at 25°C in complete medium. At mid-exponential phase, the culture was divided into seven fractions. Each fraction was incubated with chloramphenicol (0 to 600 $\mu g \cdot ml^{-1}$) for 30 min. Cells were then harvested by centrifugation and broken with glass beads as described above. Proteins were separated by SDS-PAGE on a 15% polyacrylamide slab gel overlaid with a 4% (wt/vol) polyacrylamide stacking gel. The proteins (60 μg) were boiled for 3 min in the denaturing buffer of Laemmli (27) and loaded into each well. Electrophoretic separation was performed at 120 V with a Mighty Small II SE250 apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Protein A9 was detected by immunoblotting.

RESULTS

Arrhenius plot. An Arrhenius plot (Fig. 1) was obtained by plotting the log of the specific growth rate constant μ (per hour) against the reciprocal of absolute temperature (K). The plot was linear from 20 to 7°C (the normal growth temperature range). Temperatures below 7°C decreased the slope sharply, and growth finally ceased at −5°C (data not shown). Temperatures above the normal range slowed the increase and then caused a sharp decrease in growth rate. The upper growth-limiting temperature was 32°C.

Protein synthesis patterns at 25 and 4°C. *A. globiformis* SI55 was grown at 25°C (optimal temperature) and at 4°C. Cells at each temperature were incubated in the mid-log phase with L-[^{35}S]methionine, and their protein contents were compared by two-dimensional electrophoresis. Exposing bacterial cells to radioactive amino acids for various periods is known to lead to different two-dimensional electrophoresis patterns. Cells were therefore labelled for periods corresponding to the same fraction (one-sixth) of their generation time (30 min at 25°C and 240 min at 4°C) to allow comparisons of their protein contents. The autoradiograms obtained after two-dimensional electrophoresis are shown in Fig. 2. Most proteins were similarly expressed at the two temperatures (housekeeping proteins). Eighteen peptides (numbered 1 to 18, with molecular masses of 9 to 66 kDa) presented an increased level of synthesis at the lowest temperature (Table 1) and were considered to be Caps. This term was previously used to describe proteins produced by plants at low temperatures (11). It has also been used by Roberts and Inniss (41), Whyte and Inniss (47), and Hébraud et al. (13) to describe similar proteins in psychrotrophic or psychrophilic bacteria. Seven of these Caps (A45, A24, A20, A14, B35a, C31, and E26) were poorly expressed at 25°C, while 11 (A9, A66, B16a, B16b, B19, B20, B31, C12, E13, E35, and E40) were not detected at this temperature. Nine of these Caps were overexpressed in cells growing exponentially at 4°C as well as in cells submitted to cold shocks of various magnitudes.

Growth after shocks of increasing magnitude. Cells of *A. globiformis* SI55 grown at 25°C were shifted to various colder temperatures, i.e., 15, 10, 8, 6, and 4°C. A shift from 25 to 15°C resulted in a lag period of 1 to 2 h before growth resumed at the 15°C normal growth rate. Larger shifts resulted in longer lag phases: 4 to 6 h for 25 to 10°C, 6 to 7 h for 25 to 8°C, 7 to 8 h for 25 to 6°C, and 10 to 12 h for 25 to 4°C shift (results not shown). Generation times were then similar to those measured for balanced growth at the tested temperatures (5.75 h at 15°C, 10.6 h at 10°C, 15.0 h at 8°C, 19.7 h at 6°C, and 25 h at 4°C).

Induction of Csps following temperature shifts. The two-dimensional electrophoresis protein patterns for cells subjected to temperature downshifts from 25 to 15, 10, 8, 6, and 4°C were examined after 1, 2, 4, and 6 h. All cells were incubated with L-[^{35}S]methionine for the last 30 min at the lower temperature. Each experiment was repeated four or five times. A typical autoradiogram for a 25 to 4°C shock is shown in Fig. 3. The complete results are given in Table 2. *A. globiformis* SI55 responded to cold shock by overexpressing a total of 28 proteins. The highest number of Csps was observed 4 h after the shift from 25 to 4°C and only 2 h after smaller shifts. The same set of 11 proteins was found after each shock, suggesting a common physiological response. Interestingly, nine of these proteins were Caps (A9, A14, A20, A24, A45, C12, E26, E35, and E40). Additional proteins appeared at wider shifts: 13 proteins were overexpressed when the cells were shifted from 25 to 15°C, and 26 were overexpressed after the shift from 25 to 4°C. Therefore, similar to the trend observed for other microorganisms (25), the number of Csps was correlated with the severity of the cold shock. The peptides detected following shifts from 25 to 15, 10, 8, and 6°C were all among the 26 peptides overexpressed at 4°C. Two additional proteins were absent at 4°C but overexpressed following shifts to milder temperatures: B28 was detected at 15, 10, 8, and 6°C, and C36 was detected at 15 and 10°C only.

CA. Factor maps of rows (proteins) and columns (shock magnitude) from CA are shown in Fig. 4A and B, respectively. In this CA, five factor axes (designated F1 to F5) can be used to represent the data. The two factor axes chosen maximize the variability between points. The percent variability expressed by each axis was calculated from the Eigenvalues diagram (Fig. 4C). Since the first two factor axes (F1 and F2) express almost 91% of the variability of the initial array (68% for the first axis and 23% for the second), the factor scores of these first two

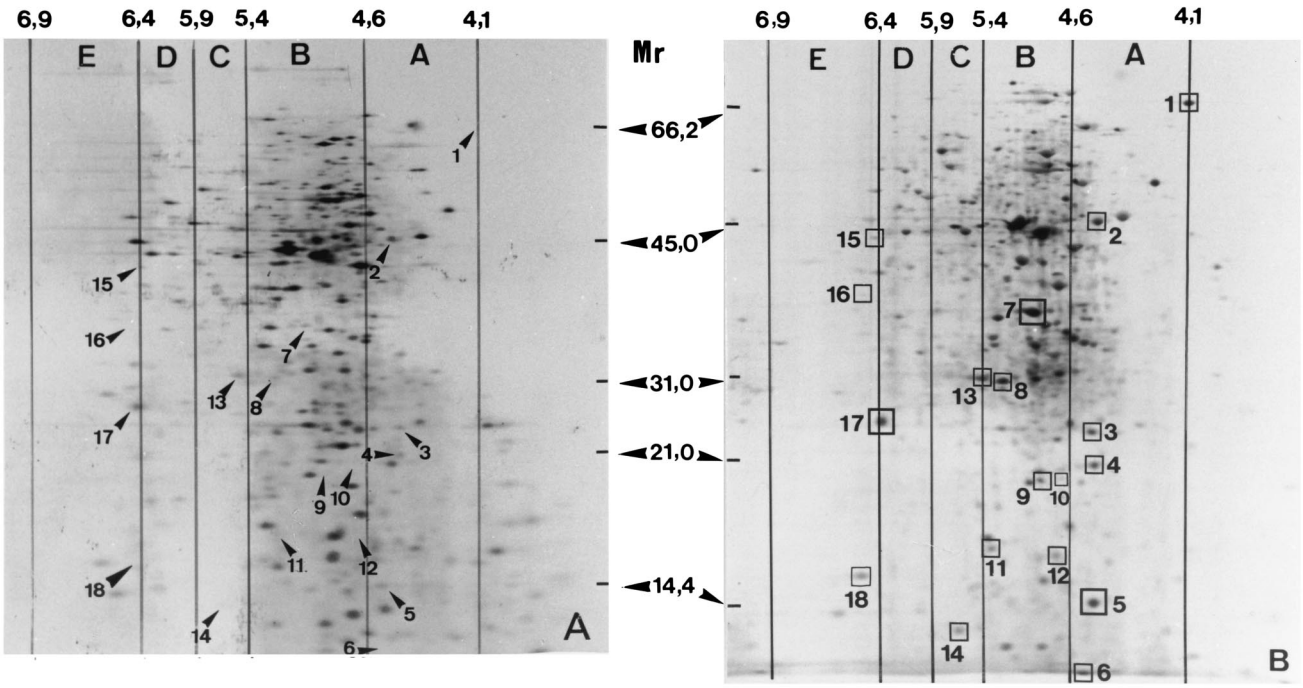

FIG. 2. Typical two-dimensional gel electrophoresis of total cellular proteins of *A. globiformis* SI55 labelled with [^{35}S]methionine during exponential growth at 25°C (A) and at 4°C (B). Each culture was labelled for one-sixth of the generation time at that temperature, i.e., 30 min at 25°C and 3 h at 4°C. Proteins overexpressed at 4°C (boxes) and their corresponding positions (arrowheads [A]) are indicated. Only proteins that were reproducibly overexpressed in five independent experiments were considered. Isoelectric points (top) and molecular weights (in thousands) are indicated.

axes of CA were used to represent the data. Proteins with similar distributions of appearance after different cold shocks occur in similar positions on the map of rows (Fig. 4A). The cold shocks inducing the occurrence of the same proteins have the same coordinates on the column map (Fig. 4B). The factor maps of rows indicate three groups of proteins (Fig. 4A), but proteins B28 and C36 could not be placed in any of these groups.

The first axis (F1) of the factor map of columns (cold shock typology) opposed the cold shock from 25 to 4°C to the four other shocks (25 to 6, 8, 10, and 15°C) (Fig. 4B). This structure did not change even when proteins B28 and C36 were excluded from the analysis. Since the two factor maps (rows and columns) are superimposable, we conclude that the group I proteins (Fig. 4A), which appeared only after a large shift (25 to 4°C), can be opposed to group III (proteins which appeared after all shifts).

The second axis (F2) of the factor map of columns (Fig. 4B) opposed the 25 to 15°C cold shock to the 25 to 6°C and 25 to 8°C cold shocks. The last two cold shocks had similar factorial scores, indicating nonsignificant differences in the protein patterns. Protein C36 appeared only after a shift from 25 to 15°C and can be opposed to several other proteins, such as protein B28, expressed mainly after the downshifts from 25 to 6°C and 25 to 8°C. Between these two extremes, the 25 to 6 or 8°C cold shocks and the 25 to 15°C cold shock order the proteins along the second axis of the CA. Therefore, the second axis of the CA contributes to the formation of group II.

TABLE 1. Summary of Caps of *A. globiformis* SI55

No.	Designation	Detection on 25°C gels	Detection after 25 to 4°C cold shock
1	A66	−	−
2	A45	+	+
3	A24	+	+
4	A20	+	+
5	A14	+	+
6	A9	−	+
7	B35a	+	−
8	B31	−	−
9	B19	−	−
10	B20	−	−
11	B16a	−	−
12	B16b	−	−
13	C31	+	−
14	C12	−	+
15	E40	−	+
16	E35	−	+
17	E26	+	+
18	E13	−	−

Detection of a *cspA*-like gene in *A. globiformis* SI55. Oligonucleotide primers based on conserved regions of the *cspA* and *cspB* genes of *E. coli* and *B. subtilis* were used to determine if *A. globiformis* SI55 possesses a gene encoding a CS7.4-like protein. Total chromosomal *A. globiformis* SI55 DNA was used as template DNA in a PCR with these primers. A band of the expected size (80 bp) on a 2% (wt/vol) agarose gel was amplified. The PCR product hybridized on Southern blots with total genomic *A. globiformis* DNA digested with several restriction enzymes (result not shown). Amplified DNA was digested with *Hin*dIII and *Eco*RI and cloned into the *Hin*dIII-*Eco*RI-digested plasmid pBluescript II-SK$^-$. Twenty-three recombinant plasmids were extracted from the *E. coli* DH5α host strain and double digested with the above endonucleases. All plasmids contained an insert of the expected size. Four of them were sequenced by using oligonucleotide probes T3 and T7 from the T7 sequencing kit. All sequences corresponded to that shown

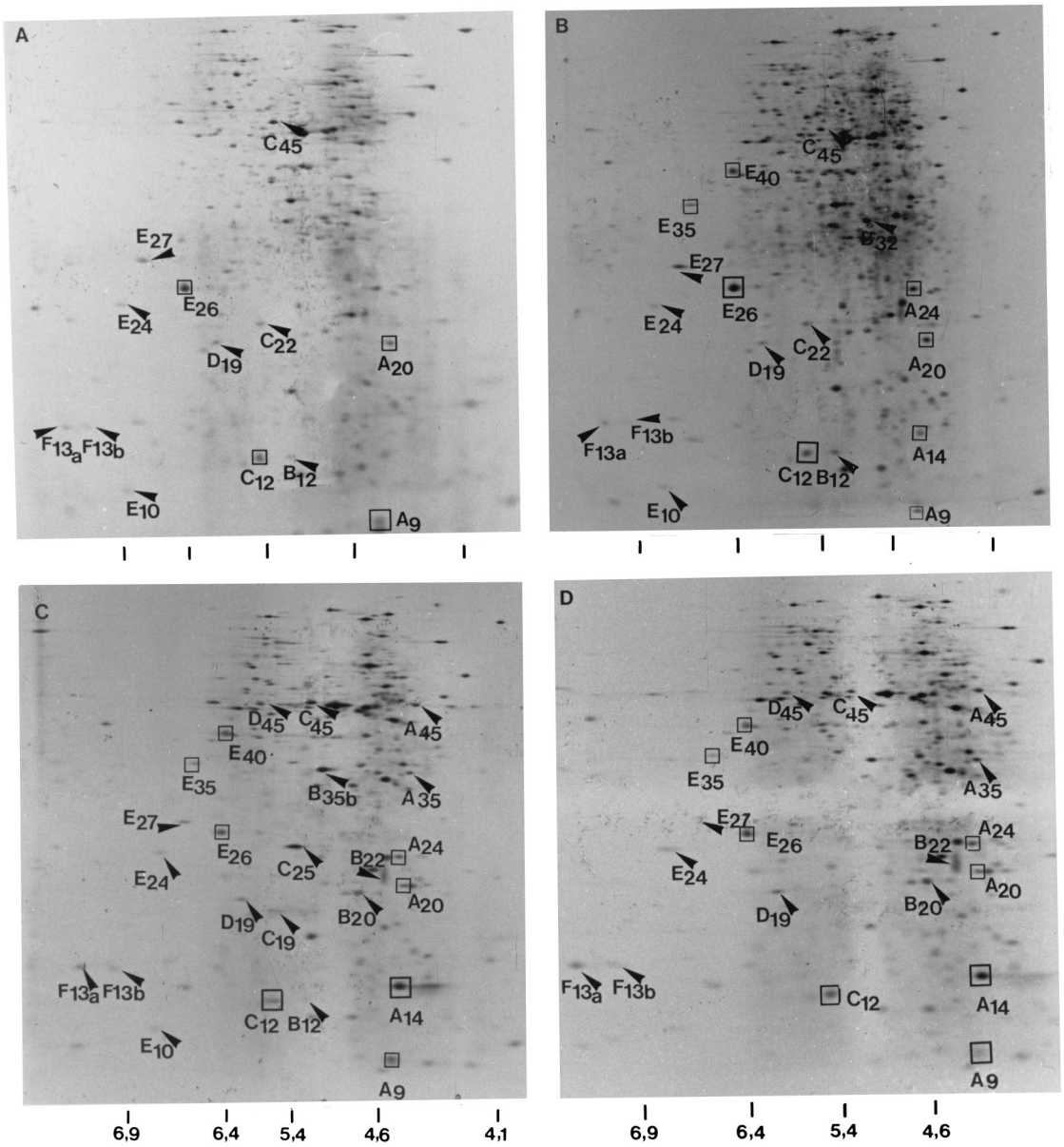

FIG. 3. Typical time course of temperature-induced protein synthesis in *A. globiformis* SI55. Cells were shifted from 25 to 4°C for 1 (A), 2 (B), 4 (C), and 6 (D) h and labelled with [^{35}S]methionine for the last 30 min of incubation. Sample preparation and two-dimensional electrophoresis were as described in Materials and Methods. Each gel was loaded with 10^6 cpm of ^{35}S-labelled TCA-insoluble material. Each electrophoretogram was compared with the protein pattern of cells grown at 25°C (Fig. 2A). Transiently overexpressed proteins (arrowheads) and proteins overexpressed both after cold shock and during prolonged growth at 4°C (boxes) are indicated. Only proteins that were reproducibly overexpressed in five independent experiments were considered. Isoelectric points are indicated at the bottom.

in Fig. 5. A peptide sequence of 18 amino acids was deduced from the DNA sequence: 10 and 14 amino acids were conserved in the corresponding sequences in *B. subtilis* CspB and *E. coli* CS7.4, respectively, suggesting that a CspB-CS7.4-like segment exists in *A. globiformis* SI55. A 14-residue peptide (Lys-Gly-Phe-Gly-Phe-Ile-Thr-Pro-Asp-Asp-Ser-Asp-Gly-Asp) corresponding to the end of the deduced peptide sequence was synthesized, covalently linked to ovalbumin, and used to immunize a rabbit. The resulting antiserum was used against *A. globiformis* SI55 proteins to detect this antigenic motif after cold shocks and to identify the corresponding protein. The peptide sequence was compared with GenBank release no. 84 by using the algorithm BLAST (1). The most similar sequence was CS7.4 from *E. coli*, followed by several other Csps of the CS7.4 family with no significant homology to other peptides (Poisson probability, <0.019). However, the antiserum recognized several proteins (Fig. 6). This nonspecific recognition was due to the fact that the antigenic motif used for immunization is common to several RNA-binding proteins (28). Only one of the immunodetected peptides, corresponding to spot A9 on autoradiograms, had an apparent molecular mass (9 kDa) and isoelectric point (4.5) similar to those reported for CspB, the homolog of CS7.4 in *B. subtilis* (48). Furthermore, it was the only peptide on the immunoblots that showed a typical CS7.4 protein induction pattern: (i) it was not produced at optimal growth temperature, and (ii) its expression was triggered by cold shock treatments or by low concentra-

TABLE 2. Proteins whose relative rates of synthesis increased following cold shocks of various magnitudes

Csp[a]	Rate of synthesis increase after the indicated cold shock[b]																			
	25 to 4°C				25 to 6°C				25 to 8°C				25 to 10°C				25 to 15°C			
	1 h	2 h	4 h	6 h	1 h	2 h	4 h	6 h	1 h	2 h	4 h	6 h	1 h	2 h	4 h	6 h	1 h	2 h	4 h	6 h
F13a	+	+	+	+	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−
F13b	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
E40	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
E35	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
E27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
E26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E24	+	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−
E10	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D45	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D19	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C45	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	+	−	−	−	−
C36	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
C25	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
C19	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
C22	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B35b	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B32	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B28	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−	−
B22	−	−	+	+	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
B20	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B12	+	+	+	−	+	+	+	−	+	+	+	−	+	+	−	−	−	−	−	−
A45	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A35	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
A24	−	+	+	+	−	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+
A20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A14	−	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	+	+	+	+
A9	+	+	+	+	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−

[a] Proteins are named according to their estimated coordinates on two-dimensional electrophoresis gels.

[b] +, increase in rate of synthesis; −, no increase in rate of synthesis. For the cold shocks from 25 to 4, 6, 8, 10, and 15°C, totals of 26, 19, 17, 14, and 13 Csps were found, respectively.

tions of chloramphenicol. The A9 protein was synthesized soon after the 25 to 4°C cold shock and was still detected in 4°C steady-state cultures. Protein A9 was also present on autoradiograms made after shifts of various magnitudes (i.e., to 6, 8, 10, and 15°C). A9 was always overexpressed 1 h after each shift.

Chloramphenicol induction of protein A9 at 25°C. *A. globiformis* SI55 cells grown at 25°C were incubated with chloramphenicol (0, 10, 30, 50, 100, 300, and 600 $\mu g \cdot ml^{-1}$) for 30 min. A9 was detected on Western blots (immunoblots) of one-dimensional electrophoresis (SDS-PAGE)-separated proteins by using the anti-CS7.4-like-motif antiserum (Fig. 7). A9 expression was strongly affected by the concentration of chloramphenicol in the medium. Low concentrations of chloramphenicol ($<30 \mu g \cdot ml^{-1}$) did not induce significant overexpression of A9 at 25°C. Concentrations of chloramphenicol from 30 to 100 $\mu g \cdot ml^{-1}$ triggered A9 production. Higher concentrations of chloramphenicol (300 and 600 $\mu g \cdot ml^{-1}$) strongly inhibited protein expression.

DISCUSSION

A. globiformis SI55 can grow at −5 to 32°C, but its optimum temperature for growth is 25°C. The linear part of the Arrhenius plot, corresponding to the normal growth range, is much narrower, spanning 20 to 7°C (Fig. 1). Temperatures below and above this range are less favorable for growth.

Protein stability is known to vary greatly with temperature. Therefore, the protein content of bacteria is expected to change with growth temperature. *E. coli* cells growing outside the normal temperature range are characterized by pronounced modifications of the levels of most proteins (15). However, we found that the two-dimensional electrophoresis protein pattern of *A. globiformis* SI55 cells growing at 25 and 4°C showed that the levels of very few proteins were altered. Most housekeeping proteins were similarly expressed at the two temperatures, but the concentrations of 18 peptides were increased in *A. globiformis* SI55 cells growing at 4°C compared with those of cells growing at 25°C (Fig. 2). Previous observations for the same bacterium (38) revealed that 13 peptides are newly synthesized or present at increased levels at 10°C. This suggests that *A. globiformis* SI55 synthesizes increasing numbers of proteins to cope with decreasing temperatures. These proteins may be involved in cold acclimation. Similar proteins, Caps, have been found in two other psychrotrophic bacteria, *B. psychrophilus* (47) and *Pseudomonas fragi* (13), grown at 0 and 4°C, respectively. These proteins may therefore be involved in maintaining some metabolic function(s) at low temperature by replacing cold-denaturated peptides. A low-temperature-specific proteolytic system has been described for *A. globiformis* SI55 (36, 37), and some of these Caps could act as cold-specific proteases that eliminate denaturated proteins whose accumulation would be deleterious for the cells. Others could be involved in maintaining membrane fluidity at low temperature or may act either as antifreeze proteins or as anabolic enzymes involved in the synthesis of putative antifreeze substances.

The synthesis of 28 peptides was increased following cold shock. Nineteen of these cold-regulated peptides were transiently overexpressed, whereas nine were still overproduced in

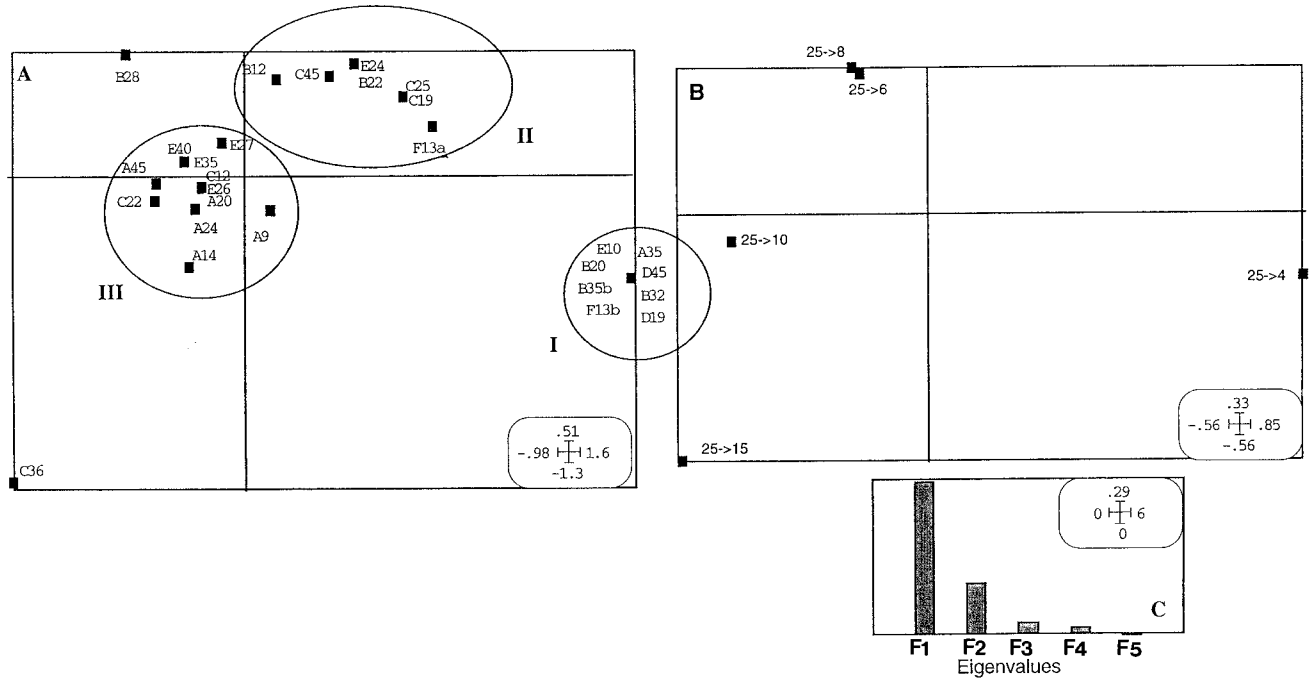

FIG. 4. CA of protein occurrence distributions in five cold shocks with different magnitudes. CA was performed by using a transformed set of data obtained from Table 2 as described in the text. (A) Factor map of rows (i.e., proteins); the proteins with similar distributions of appearance in the different cold shocks occur in similar positions on the map. (B) Factor map of columns (i.e., cold shock magnitude); the cold shocks inducing the occurrence of the same proteins have the same coordinates on the map. (C) Eigenvalues diagram. This diagram shows that only the first two axes, F1 and F2, need to be considered (see text for details).

4°C steady-state cells. As suggested by Hébraud et al. (13), the term "Csps" is used here for proteins that are transiently overexpressed, and the term "Caps" is used for proteins whose production is increased continuously in cells adapted to the low temperature. While the distinction between these two classes of proteins is obvious, a variability in the kinetics of induction of individual peptides within each class may exist. Mathematical analysis of the results by CA was used to search for additional subdivisions. This analysis clearly demonstrated the existence of three distinct groups of proteins. Group I corresponds to Csps that are specific to the 25 to 4°C shock, group II includes Csps that also appear after mild shocks (except 25 to 15°C), and group III contains proteins overexpressed after all the shocks. This third group includes some of the proteins previously identified as Caps and which can therefore be referred to as early Caps. A fourth group includes the Caps that are not overexpressed during the first stages of acclimation to cold and that were therefore not included in the mathematical analysis; they are late Caps.

The analysis also indicates that the temperature shifts fall into three categories: (i) the 25 to 15°C shock, (ii) the 25 to 10, 8, and 6°C shocks (mild shocks), and (iii) the 25 to 4°C shock, which is distinct from all the other cold shocks. The first shock (25 to 15°C) is characterized by the reduced number of overexpressed proteins as well as a very reduced lag phase. Since it does not cause large changes in gene expression, it is probably not a stress. This transfer temperature corresponds to the physiological optimum of *A. globiformis* SI55 as indicated by the Arrhenius plot. Cold shocks are known to induce modifications in DNA superhelicity (16) that are susceptible to modification of the access of RNA polymerase to several promoters (40). Therefore, overexpression of some proteins can be partly explained by the fact that a small set of genes might be turned on by the temperature decrease, the products of which are not involved in any adaptation process. They would therefore appear as early Caps. If proteins C36 and B28, which are atypical, are ignored, 8 of the 11 proteins detected at 15°C are effectively early Caps (group III).

A

```
CTT CAA CGC TGA AAA GGG CTT CGG CTT CAT CAC CCC GGA TGA CTC CGA TGG CGA
GAA GTT GCG ACT TTT CCC GAA GCC GAA GTA GTG GGG CCT ACT GAG GCT ACC GCT
```

B

```
                                   RNP-1 motif
                                   <---------->
CspAgl              ...F N A E K G F G F I T P D D S D G D ...
CS7,4 (CspA)        ...- - - D - - - - - - - - - G S K - ...
CspB                ...- - S - - - - - - - E V : E G Q D - ...
```

FIG. 5. (A) Sequence of the PCR-amplified region determined by using *A. globiformis* SI55 genomic DNA as a matrix. (B) Comparison of the deduced amino acid sequence of the PCR-amplified fragment with the corresponding regions of *B. subtilis* CspB and *E. coli* CS7.4. Amino acids identical to those of the corresponding gene product (CspAgl) in *A. globiformis* SI55 are indicated (dashes).

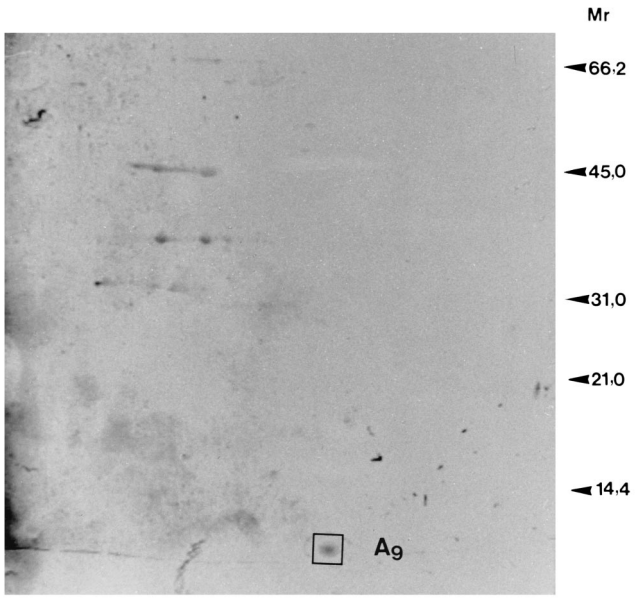

FIG. 6. Western blot of *A. globiformis* SI55 cellular proteins with a synthetic-peptide antiserum (anti-CS7.4-like motif). Exponentially growing cells at 25°C were shifted to 4°C for 4 h, and total proteins were separated by two-dimensional electrophoresis. Immunochemical analysis was performed as described in Materials and Methods. The boxed protein corresponds to protein 6 (A9) in Fig. 2B. Molecular weights (in thousands) are indicated on the right.

Mild shocks (from 25 to 6, 8, and 10°C) cause a lag phase and a number of overexpressed proteins, both of which increase with the severity of the shock. This result agrees with those of Julseth and Inniss (25) for the psychrotrophic yeast *T. pullulans* showing that the number of cold-regulated proteins increased with the magnitude of the shock. Proteins overexpressed following mild shocks belong to group II Csps (and of course to group III, as discussed above). These shocks seem to produce a moderate stress that requires the transient synthesis of a limited number of Csps before growth resumes at the new temperature.

The 25 to 4°C cold shock is followed by a long lag phase (10 to 12 h). It is also characterized by the appearance of a specific set of Csps (group I) in addition to the group II and III proteins. It is therefore a severe stress, although 4°C is far from the minimal growth temperature for *A. globiformis* SI55. As for the heat shock response, the synthesis of these proteins could be initiated by activation of a particular operon under harsh

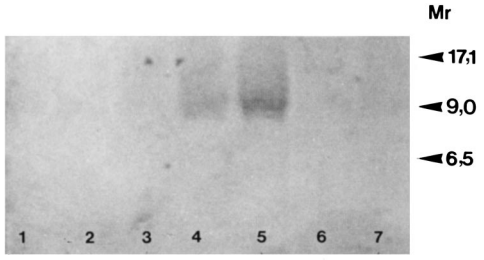

FIG. 7. Induction of A9 (CS7.4-like protein) at 25°C by chloramphenicol. Exponentially growing cells at 25°C were treated for 30 min with increasing concentrations of chloramphenicol. A9 was detected on Western blots of total cellular proteins separated by SDS-PAGE as described in Materials and Methods. Lane 1, no chloramphenicol; lanes 2 to 7, chloramphenicol at 10, 30, 50, 100, 300, and 600 $\mu g \cdot ml^{-1}$, respectively.

conditions. Csps belonging to group I seem to be stress proteins because they seem specifically required for drastic shocks. These Csps may act directly and specifically after a cold shock to prepare the cells for growth at the new temperature, for example, by initiating the synthesis of late Caps, and/or to repair damage due to the cold. Although there are very few data on the cold shock response in psychrotrophic bacteria, more Csps seem to be overexpressed in psychrotrophs than in mesophilic bacteria. Julseth and Inniss (25) reported the induction of 26 Csps after a 24 to 5°C cold shock in the psychrotrophic yeast *T. pullulans*. Cloutier et al. (5) showed that arctic *Rhizobium* strains respond to a very low temperature (−10°C) by synthesizing more proteins than temperate strains do at higher temperatures. In contrast to the situation in cold-loving microorganisms, fewer Csps are found in the mesophilic bacterium *E. coli* (24), since the levels of only 14 polypeptides are increased. In *Lactococcus lactis* cells shifted from 30 to 8°C, a dozen proteins are overexpressed up to threefold in comparison with cells growing at 30°C (34). These findings suggest that the cold shock response of mesophilic bacteria might be incomplete and could be only a part of the total response of cold-adapted bacteria. This would explain the inability of the mesophilic cold shock response to restore harmonious growth at low temperatures.

Protein CS7.4, the *cspA* gene product, is the most abundant cold shock protein in *E. coli* after a shift from 37 to 10°C (9). It shares 61% identity with CspB of *B. subtilis* (48) and 56% identity with SC7.0 from *S. clavuligerus* (2). The major cold shock proteins of mesophilic bacteria therefore seem to be highly conserved. A similar gene was looked for in *A. globiformis* SI55 by using PCR. This cold-adapted microorganism does possess a *cspA*-like motif. Its peptide sequence showed 78% identity with the corresponding region of CS7.4 and 56% identity with CspB. Like the corresponding proteins in mesophilic bacteria, it contains an RNP1 motif, which suggests that it might act as a DNA- and/or RNA-binding protein. A synthetic peptide-directed antibody detected a CS7.4-like protein on two-dimensional electrophoresis gels. This corresponds to protein A9, which is a class III protein (early Caps) in our analysis. Unlike the case in mesophilic organisms, in which this protein is transiently synthesized after a cold shock, A9 was still produced during prolonged growth at low temperature. In addition, the level of A9 induction appeared to be much lower than that of the corresponding protein in *E. coli*, whose synthesis increases 200-fold 2 h after a shift from 37 to 10°C. We therefore assume that, in contrast to CS7.4, A9 might be required at lower levels during cold shocks in *A. globiformis* SI55, and it might serve some particular adaptive function necessary for prolonged growth at low temperature. Cold may also have a deleterious effect on the control mechanism that regulates the expression of CS7.4 in *E. coli*. The diversion of the translational machinery for the preferential production of CS7.4 could therefore block the synthesis of other proteins. Thus, the cold shock response of mesophilic bacteria appears to be the manifestation of a deregulated adaptive mechanism that allows psychrotrophs to acclimate to cold.

Van Bogelen and Neidhardt (45) showed that the cold shock response can be triggered in *E. coli* at high temperatures by various C group antibiotics (chloramphenicol, tetracycline, erythromycin, spiramycin, and fusidic acid). They suggested that the ribosome is the primary sensor of conditions that elicit the cold shock response in *E. coli*. However, Jiang et al. (21) showed that the induction of CS7.4 synthesis by low concentrations of chloramphenicol was regulated at the transcriptional level. Similarly, we found that the expression of A9 can be triggered at 25°C by chloramphenicol concentrations rang-

ing from 30 to 100 $\mu g \cdot ml^{-1}$. Hence, we speculate that the expression of CS7.4-like proteins is not regulated by the absolute temperature, and the same biochemical event may occur at a relatively high temperature for a mesophile and at lower temperatures for a psychrotroph. This is supported by the existence of CS7.4-like proteins in thermophilic bacilli (43). The important event in the induction of these proteins might involve the effects of cold, as well as other factors, on the controlling elements which determine the activation of the relevant genes. This suggests a more general role of CS7.4-like proteins than their sole implication in cold acclimation processes.

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